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Characterization of potent antidiabetic compounds from Costus pictus D. Don found in Assam, India using in vitro in vivo methods

Caracterización de compuestos antidiabéticos potentes de Costus pictus D. Don encontrados en Assam, India usando métodos in vitro e in vivo

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ABSTRACT

Objective: *Costus pictus* D. Don is a traditionally used plant in Naojan area of Golaghat district of Assam, India specifically for treating diabetes. Six compounds were isolated from standardized methanolic extract of the aerial parts (MECP). The prime objective was to select most potent antidiabetic compounds among the isolated compounds viz. F67, F12, F16, F3032, F37 & F48 by using *in vitro* and *in vivo* methods.

Methods: Isolated compounds were subjected to initial screening by *in vitro* α-amylase inhibition activity assay using iodine-starch and DNSA (3,5-dinitrosalicylic acid) methods. Compounds depicting promising *in vitro* activity were selected for *in vivo* Streptozotocin (STZ) induced antidiabetic screening activity. Then based on the i*n vivo* results, most potent compounds were selected for instrumental characterization by Q-TOF ESI-MS, 1HNMR, 13CNMR & FTIR.

Results: Amongst the six compounds isolated from MECP, three compounds *viz.* F12, F16 & F48 showed potent *in vitro* activity. They were subsequently subjected to evaluation of the antidiabetic activity *in vivo* by oral administration, at dose of 10, 20 & 50 mg/kg body weight respectively, using Wister rat (120-150 g) and Glibenclamide (10mg/k body weight) as standard. Two compounds, F12 and F48 at dose of 50 mg/kg body weight, reversed STZ induced diabetic parameters (increased blood glucose level, altered plasma profile and histoarchitecture of the pancreatic and hepatic cells) with statistical significance ($P<0.05$), that was comparable with the standard. Hence, instrumental characterization by Q-TOF ESI-MS, 1HNMR, 13CNMR & FTIR of compounds F12 and F48 isolated from MECP was carried out which established their identity as (3,5,7-Trihydroxy-3'-hydroxy-4'-methoxy) flavanone or [3,5,7-Trihydroxy-2-(3'-hydroxy-4'-methoxy phenyl)-2,3-dihydrochromen-4-one] and 3,5,8-trihydroxy-7-methoxy-2-phenyl-2,3-dihydrochromen-4-one or [7-methoxy-3, 5, 8 trihydroxy flavanone] respectively.

Conclusion: The study culminated in elucidation of two flavanones as most potent compounds in exhibiting antidiabetic activities. The findings were thus successful in validating the traditional practices in Golaghat district of Assam, India, associated with the use of *Costus pictus* D. Don in the treatment of diabetes.

Keywords: *Costus pictus*; antidiabetic activity; trihydroxyflavanone

RESUMEN

Objetivo: *Costus pictus* D. Don es una planta usada tradicionalmente en la zona del distrito de Golaghat Naojan de Assam, India específicamente para el tratamiento de la diabetes. Seis compuestos se aislaron a partir de extracto metanólico estandarizados de las hojas (MECP). El principal objetivo fue seleccionar compuestos antidiabéticos más potentes entre los compuestos aislados viz. F67, F12, F16, F3032, F37 y F48 mediante métodos *in vitro e in vivo.*

Métodos: Los compuestos aislados fueron sometidos a cribado inicial mediante ensayo de actividad de inhibición *in vitro* α-amilasa utilizando yodo-almidón y métodos DNSA (ácido 3,5-dinitrosalicilico). Los compuestos que presentaban una actividad *in vitro* prometedora se seleccionaron para la actividad de

cribado antidiabético inducida por estreptozotocina (STZ) *in vivo*. En función de los resultados *in vivo*, la mayoría de los compuestos potentes se seleccionaron para la caracterización instrumental por Q-TOF ESI-MS, 1HNMR, 13CNMR y FTIR.

Resultados: Entre los seis compuestos aislados de MECP, tres compuestos viz. F12, F16 y F48 mostraron una potente actividad. Posteriormente se sometieron a evaluación de la actividad antidiabética *in vivo* mediante administración oral, en dosis de 10, 20 y 50 mg / kg peso, utilizando ratas Wister (120-150 g) y glibenclamida (10 mg / kg peso) como estándar. Dos compuestos, F12 y F48 en dosis de 50 mg/kg peso, revirtieron los parámetros diabéticos inducidos por STZ (aumento del nivel de glucosa en sangre, plasma perfil alterado y histoarquitectura del páncreas y células hepáticas), con significación estadística (P<0,05) que era comparable con la norma. Se llevo a cabo la caracterización instrumental por Q-TOF ESI-MS, RMN 1H, RMN 13C y FTIR de los compuestos F12 y F48 aislado de MECP, lo que estableció su identidad como (3,5,7-trihidroxi-3 '-hidroxi-4'- metoxi) flavanona o [3,5,7-trihidroxi-2- (fenil 3 '-hidroxi-4'-metoxi) -2,3-dihydrochromen-4-ona] y 3,5,8-trihidroxi-7-metoxi -2-fenil-2,3-dihydrochromen-4-ona o [7-metoxi-3, 5, 8 trihidroxi flavanona] respectivamente.

Conclusión: El estudio culminó en la elucidación de dos flavanonas como los compuestos más potentes en la exposición de actividades antidiabéticas. Los resultados lograron validar las prácticas tradicionales en el distrito de Golaghat de Assam, India, asociados con el uso de *Costus pictus* D. Don en el tratamiento de la diabetes.

Palabras clave: *Costus pictus*; Actividad antidiabética; trihydroxyflavanone

INTRODUCTION

Costus pictus D. Don belongs to the genus Costus pertaining to the family Costaceae[1]. This plant is mainly distributed in the neo tropical regions[2]. In India it found in the sub-Himalayan tract from Himachal Pradesh to Arunachal Pradesh; and in the Western Ghats in Goa, Kerala and Tamil Nadu[3]. The presence of *Costus pictus* in Assam and its usage in traditional medicine is the first report by the authors from this part of India[4]. It is an erect plant, up to 3 meters high; root stock is tuberous; stem is sub-woody at the base. Leaves are elliptical and are spirally arranged around the stem. Leaf surface is firm and leathery. The primary bracts are borne on the inflorescence in spiral phyllotaxy. Flowers are yellow in colour with pink radiating stripes originating from the base. Flowering time in Indian condition is August to October.

Aerial parts of the plant are extensively used in Golaghat district of Assam by the traditional healers for the treatment of diabetes[4-6].

The study was contemplated with the main objective of validation of the traditional usage and to identify most potent antidiabetic compounds by using *in vitro* and *in vivo* methods.

MATERIALS AND METHODS

Reagents and Instruments Used

AR and GR grade reagent and chemicals purchased from Renkem, Hi Media, Merck, SRL etc. were used for performing different procedures in the study. Instruments like Bruker FTIR, Leica DM 1000 Photomicroscope, Zeiss Projection Microscope, Lab India- UV-3200 UV Spectrophotometer etc. were used in different stages of the experimental study.

Collection of Plant Materials

Aerial parts of *Costus pictus* were collected from Naojan area of Golaghat district of Assam, India during the month of September-October.

Authentication of Plant Materials

Plant specimen herbariums were sent to Ministry of Environment and Forests, Government of India, Botanical Survey of India, Eastern Regional Centre, Shillong, Meghalaya for authentication. The plant specimen was identified and confirmed as *Costus pictus* D. Don, Family: Costaceae by Dr. R.K. Singh, Scientist-D and HOD vide letter no. BSI/ERC/Tech./2015-16.

Preliminary Processing of Plant Materials

The collected plant parts i.e. aerial parts of *Costus pictus* were washed thoroughly under running water to remove all adhering soil particles. After cleaning, the crude drug materials were dried under shed for about 6 week time. Then the crude drug materials were subjected to size reduction in a blender and packed in air tight containers for further study.

Isolation of Compounds from Standardized Plant Extracts

The dried crude drugs were extracted with methanol in Soxhlet apparatus. The methanolic extract of *Costus pictus* (MECP) was then subjected to column chromatography using Silica gel (120 mesh) as stationary phase for the isolation of the phytoconstituents. The mobile phases used were in various ratios in increasing order of polarity, after repeated washing with DCM (dichloromethane) and petroleum ether to remove chlorophyll type impurities.

In vitro Screening of Compounds Based on Anti Diabetic Activity

α-amylase inhibition activity of isolated compounds were assayed to screen compounds with potent anti diabetic property. Two methods *viz.* 3, 5-dinitrosalicylic acid

(DNSA) inhibition assay and Iodine-Starch method was used to do the screening.

DNSA Inhibition Assay Method

The α-amylase activity is measured using a colorimetric method with 3, 5-dinitrosalicylic acid (DNSA) reagent. In this method, starch is converted into maltose by α -amylase. Maltose released from starch is measured by the reduction of 3, 5-dinitrosalicylic acid. Maltose reduces the pale yellow coloured alkaline DNSA to orange- red colour. The intensity of the colour is proportional to the concentration of maltose present in the sample and is measured using a spectrophotometer at 540nm wavelength[7].

Various concentrations (1, 2, 4, 8, 10 and 12 µg/ml) of components, isolated from MECP were prepared in 10%DMSO[8]. 0.5 ml of each concentration was added to 0.5 ml of 0.02M sodium phosphate buffer (pH 6.9 having 6mM sodium chloride) containing 0.04 unit of α-amylase and incubated at 37°C for 10 min. Then 0.5ml 1% starch solution prepared in 0.02M sodium phosphate buffer (pH 6.9) was added after which 1ml DNSA reagent was added to each and put in boiling water bath for 5min. After that the tubes were removed and allowed to cool to room temperature. 10ml distilled water was added and absorbance was measured at 540nm using UV-Visible spectrophotometer. The control was prepared by the same procedure but, without adding any sample. The results were expressed as % inhibition calculated using the formula:

% inhibition = $[(A_{control} - A_{test}) / A_{control}] \times 100$, Where, $A_{control}$ = absorbance of the blank control (containing all reagents except the test solution), A_{test} =absorbance of the test sample.

A percent inhibition versus concentration curve was then plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

Iodine Starch Method

The inhibition assay was performed according to Xiao *et al* (2006)[9] using iodine-starch method. Various concentrations (1, 2, 4, 8, 10 and 12 µg/ml) of components, isolated from MECP were prepared in 10%DMSO[8]. 0.5 ml of each concentration was added to 0.5 ml of 0.02M sodium phosphate buffer (pH 6.9 having 6mM sodium chloride) containing 0.04 unit of α –amylase and incubated at 37°C for 10 min. Then 0.5ml 1% starch solution prepared in 0.02M sodium phosphate buffer (pH 6.9) was added and incubated again at 37°C for 15 min. Then one drop of 1M HCl and 0.1 ml of iodine reagent (5mM I_2 + 5mM KI) was added. Then 10ml distilled water was added and absorbance was measured at 620nm using UV-Visible spectrophotometer. The control was prepared by the same procedure but, without adding any sample. The results were expressed as % inhibition calculated using the formula:

% inhibition = $[(A_{control} - A_{test}) / A_{control}] \times 100$, Where, $A_{control}$ = absorbance of the blank control (containing all reagents except the test solution), A_{test} =absorbance of the test sample.

A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

In vivo Anti Diabetic Activity Study of Screened Compounds

Based on the *in vitro* results the screened compounds were considered for *in vivo* evaluation of anti diabetic activity.

Toxicity Studies

The toxicity study was aimed at establishing the therapeutic index i.e. the ratio between the pharmacologically effective dose and the lethal dose, and also to perform the primary screening.

Toxicity studies were conducted for MECP as per protocol drawn under OECD guidelines 425 in female Wister rats. Limit test at 2000 mg/kg was conducted as per section 23 of OECD guideline 425 and were found to be safe up to a dose of 2000 mg/kg body weight. Three dose levels were selected for the isolated compounds *i.e.* 10, 20 and 50 mg/kg body weight (oral administration*)*. based on the toxicity study.

Grouping of Animals

Healthy Wister rats weighing between 120-150 gm were selected for performing the *in vivo* study. Animals were fed on standard laboratory diet with water *ad libitum* and housed in plastic cages at room temperature. The formulated diet contained corn flour, dry fish, wheat flour and multivitamin syrup, which were mixed in equal ratio to form dough and then made into pellets and dried.

The duration for the experimentation procedure was three weeks.

Induction of Diabetes

Diabetes was induced in the rats by single intraperitonial injection (*i.p.*) of streptozotocin (STZ), prepared by dissolving in citrate buffer (pH 4.5), at a dose of 60 mg/kg body weight along with nicotinamide (NIC) at a dose of 120 mg/kg body weight after overnight fasting. Animals with FBS≥200mg/dl were considered for the study.

Treatment Regimen of Experimental Animals

Animals were treated accordingly as per the following regimen:

1. 1. Group I: Normal Control, treated with vehicle i.e. 0.3% CMC (carboxy methyl cellulose) suspension at a dose of 10 ml/kg body weight
2. 2. Group II: Negative Control, treated with STZ (60 mg/kg body weight; *i.p.*) and NIC (120 mg/kg body weight; oral administration). Here, *i.p.* stands for intraperitonial route of administration.
3. 3. Group III: Standard, treated with (STZ+NIC)+Glibenclamide, (10 mg/kg body weight; oral administration)
4. 4. Group IV: Test Dose F12.1, F16.1, F48.1, treated with (STZ+NIC) +10mg/kg of compound F12, F16 and F48.
5. 5. Group V: Test Dose F12.2, F16.2, F48.2, treated with (STZ+NIC) +20mg/kg body weight; oral administration of compound F12, F16 and F48.
6. 6. Group VI: Test Dose F12.3, F16.3, F48.3, treated with (STZ+NIC) +50mg/kg body weight; oral administration of compound F12, F16 and F48.

Blood glucose level (FBS) and plasma lipid profile was examined on 1st, 7th, 14th and 21st day one hour after treatment. On the last day, one hour after the treatment animals were sacrificed followed by isolation of pancreas and liver for histopathological examination. The FBS was recorded using Accu-Chek ® Active blood glucose monitoring system.

Evaluation of Serum Biochemical Parameter

Blood was collected and allowed to stand for 20 min, and then centrifuged for 15-20 minutes at 2000 rpm to separate the serum and the latter was used for serum biochemical parameters (total cholesterol, triglycerides, ALT, AST and ALP).

Total Cholesterol Estimation

Total Cholesterol was estimated using CHOD/POD method[10]. The absorbance of the standard and test sample against blank was measured at a wavelength of 505 nm. Total Cholesterol content was then measured using the following formula,

Cholesterol (mg/dl) = (Abs. T/Abs. S) x 200 mg/dl; where T denotes test, S denotes standard and Abs denotes absorbance. 200 mg/dl is the concentration of standard used.

Serum Triglyceride Estimation

Serum Triglyceride was estimated using GPO or GOD-POD method[11]. The absorbance of the standard and test sample against blank was measured at a wavelength of 500 nm. Triglycerides content was then measured using the following formula,

Triglycerides (mg/dl) = (Abs. T/Abs. S) x 200 mg/dl; where T denotes test, S denotes standard and Abs denotes absorbance. 200 mg/dl is the concentration of standard used.

Serum ALT and AST Estimation

Serum ALT and AST were estimated at 37^{0}C by colorimetric method[12] at 505 nm using 2, 4 DNPH. The enzyme activity was directly calculated from calibration curve prepared with standard.

Estimation of Serum ALP

Alkaline Phosphatase (ALP) is also measured using colorimetric method[13] at 507nm and 37^{0}C at an alkaline pH. The intensity of the colour is directly proportional to the activity of ALP present in the sample.

The enzyme activity was calculated using the following formula,

Total ALP activity in K.A. units = (Abs. T – Abs. C/ Abs. S – Abs. B) x 10 where T is test, C is control, S is standard, B is blank and Abs is absorbance. Conversion, 1K.A. unit = 7.1 U/L

Histopathological Studies

On the last day, one hour after the treatment, animals were sacrificed followed by isolation of pancreas and liver for histopathological examination. Isolated samples were preserved at 2-3°C with Bouin's solution. For the histopathological examination samples were prepared using Rapid process. The samples were cut into thin ribbon using rotary microtome and strips placed in glass slides. These slides were then incubated overnight at a temperature of 37°C. After treatment with xylene and alcohol the slides were finally washed with water for 20min. The slides were stained with haematoxylin and eosin, after which it was fixed and observed under microscope (Leica Photomicroscope DM1000)[14,15].

Instrumental Characterization of Selected Potent Compounds

The most potent compounds were selected for instrumental characterization followed by structure elucidation based upon the result generated after following *in vitro* and *in vivo* procedure as described above. 13C NMR, 1H NMR, FT-IR and MS spectral analysis was carried out for the potent compounds at Sophisticated Analytical Instrumentation Facility (SAIF), Punjab University, Chandigarh.

RESULTS AND DISCUSSION

Extraction and Isolation of Compounds

The processed plant drug materials were subjected to hot extraction using methanol as solvent by Soxhlet apparatus. The extract so obtained was termed as methanolic extract of *Costus pictus* D. Don (MECP).

Column chromatography of MECP was performed using different solvent systems and a total of six compounds *viz.* F67, F12, F16, F3032, F37 and F48 were isolated after suitable TLC characterization.

Screening of Compounds Based on In Vitro α-Amylase Inhibition Activity

DNSA Inhibition Assay

The DNSA inhibition assay was done according to the method of Miller *et. al* [7]. The concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value. Three compounds *viz.* F12, F16 and F48 exhibited IC_{50} value of 10.16, 4.30 and 3.25 µg/ml respectively (Table 1).

Table 1. IC_{50} values of compounds isolated from MECP*

Compounds	IC_{50} value (µg/ml) (DNSA inhibition method)	IC_{50} value (µg/ml) (Iodine-Starch method)
F67	--	--
F12	10.16	11.69
F16	4.30	8.90
F3032	--	--
F37	--	--
F48	3.25	5.57

*MECP is methanolic extract of *Costus pictus* D. Don

Iodine-Starch Method

The Iodine-Starch method was done according to the method of Xiao *et. al* [9]. The concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value. Three compounds *viz.* F12, F16 and F48 exhibited IC_{50} value of 11.69, 8.90 and 5.57 µg/ml respectively (Table 1).

Evaluation of In Vivo Anti Diabetic Activity of Screened Compounds

Streptozotocin induced diabetic rat model using three doses i.e. 10, 20 and 50 mg/kg body weight; oral administration, daily was used for *in vivo* antidiabetic activity evaluation. The *in vivo* study was approved by IAEC (Institutional Animal Ethical Committee) of Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh, Assam-786004 (Regd. No. 1576/GO/a/ 11/CPCSEA Dated: 17/02/2012) vide approval No: IAEC/DU/36 Dated: 03/12/2012. The fasting blood sugar of the experimental animals was measured on the 1st, 7th, 14th and 21st day of the study. The results are depicted in Table 2.

Serum Biochemical Parameter Evaluation

Serum biochemical parameters such as total cholesterol, triglyceride, AST, ALP and ALT levels were measured and tabulated in Table 3. The results so obtained were then subjected to statistical analysis.

Histopathological Study

Histopathological studies on liver and pancreas obtained from animals belonging to different groups were conducted as per the above discussed procedure and are represented in Figure 1 and 2.

Different compounds isolated from MECP were found to alter the biomarker levels and the 50 mg/kg body weight; dose closely resembles to that of the standard group in successfully controlling the elevated cholesterol and triglyceride levels. Group F16.3 and F 48.3 treated with 50mg/kg animals significantly reversed the STZ induced diabetic parameters (increased blood glucose level, altered serum profile and histoarchitecture of the pancreatic and hepatic cells) that is comparable with the standard drug. It can therefore be concluded that groups TDF12, TDF16 & TDF48 has significant role in reversing altered lipid profile but the group TDF16 and 48, particularly at the dose 50mg/kg has been found to be more effective in successfully reversing the diabetic parameters induced by STZ. Hence, compounds F16 and F48 isolated from MECP were considered for further instrumental characterization and structure elucidation.

Table 2. Effects of different doses of compounds isolated from MECP on blood glucose levels in the treatment period

Animal Group		Blood glucose concentration in mg/dl			
		1st day	7th day	14th day	21st day
Group I Normal Control		91.75 ±2.59	93.49 ±2.17	90.91 ±3.54	92.55 ±2.33
Group II Negative Control		317.38 ±14.94	323.65 ±11.56###	319.71 ±13.82###	321.85 ±11.28###
Group III Standard Treated		302.33 ±18.51	231.54 ±6.36***	186.58 ±7.73***	143.21 ±10.01***###
Group IV Test Gr 1 (10mg/kg)	F12.1	314.35 ±13.42	287.16 ±9.69***###	278.33 ±8.31***###	268.14 ±8.53***###
	F16.1	309.78 ±12.38	294.44 ±10.30***###	286.82 ±5.47***###	277.47 ±7.41***###
	F48.1	336.07 ±18.66###	301.81 ±10.16***###	278.38 ±8.45***###	263.47 ±9.55***###
Group V Test Gr 2 (20mg/kg)	F12.2	320.11 ±12.73	277.92 ±6.31***###	250.09 ±4.19***###	237.32 ±5.87***###
	F16.2	311.71 ±12.34	284.47 ±7.46***###	267.54 ±8.14***###	258.07 ±9.83***###
	F48.2	318.97 ±11.07	281.53 ±5.06***###	273.01 ±7.43***###	266.52 ±5.43***###
Group VI Test Gr 3 (50mg/kg)	F12.3	313.13 ±9.36	268.77 ±6.87***###	246.31 ±8.55***###	213.61 ±7.07***###
	F16.3	308.07 ±14.33	259.15 ±4.88***###	236.61 ±6.33***###	192.81 ±3.41***###
	F48.3	307.46 ±15.60	233.84 ±9.04***	197.74 ±5.91***	157.12 ±8.81***#

Values are expressed as mean ± SD (number of animals, n=6) significantly different at *p<0.1,**p<0.01, ***p<0.001 using Dunnett Compare all vs negative control and at #p<0.1, ##p<0.01, ###p<0.001 using Dunnett Comapare all vs standard treated.

Table 3. Effects of different doses of compounds isolated from MECP on lipid profile and biochemical parameters in the treatment period

Animal Group		Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	ALT (U/L)	AST (U/L)	ALP (U/L)
Group I Normal Control		108.33±9.72	123.75±5.96	24.09±3.41	21.63±5.50	133.89±7.06
Group II Negative Control		489.75±16.13 ###	267.41±9.74###	68.11±7.34###	64.73±4.33###	177.54±8.93###
Group III Standard Treated		143.62±7.13***	164.89±6.73***	33.86±7.51***###	29.74±6.13***	139.77±6.03***
Group IV Test Gr1 (10mg/kg)	F12.1	238.14±11.03***###	263.17±4.79###	59.37±7.43###	53.32±6.19*###	151.54±9.44***
	F16.1	211.39±6.74***###	234.63±8.34***###	53.73±8.11**###	56.75±7.27###	164.11±8.75*###
	F48.1	156.57±6.31***	189.91±3.17***###	50.61±7.29***##	42.59±6.26***##	141.45±5.31***
Group V Test Gr 2 (20mg/kg)	F12.2	218.57±7.29***###	247.56±5.60***###	57.31±6.37*###	44.43±4.73***##	144.57±8.43***
	F16.2	201.05±4.76***###	216.77±8.65***###	49.17±7.34***###	50.03±7.72**###	158.13±4.57***###
	F48.2	143.91±3.4***	181.89±7.75***##	45.75±4.63***#	39.01±6.23***	143.57±6.31***

Group VI Test Gr 3 (50mg/kg)	F12.3	187.53±9.23***###	238.91±6.13***###	54.07±6.35**###	41.71±3.74***#	131.08±8.48***
	F16.3	179.11±6.15***###	202.03±11.44***###	46.16±4.78***#	43.28±6.16***##	140.54±6.11***
	F48.3	151.13±4.89***	173.755±1.04***	39.61±3.12***	27.43±9.11***	133.71±7.91***

Values are expressed as mean ± SD (number of animals, n=6) significantly different at *p<0.1,**p<0.01, ***p<0.001 using Dunnett Compare all vs negative control and at #p<0.1, ##p<0.01, ###p<0.001 using Dunnett Comapare all vs standard treated.

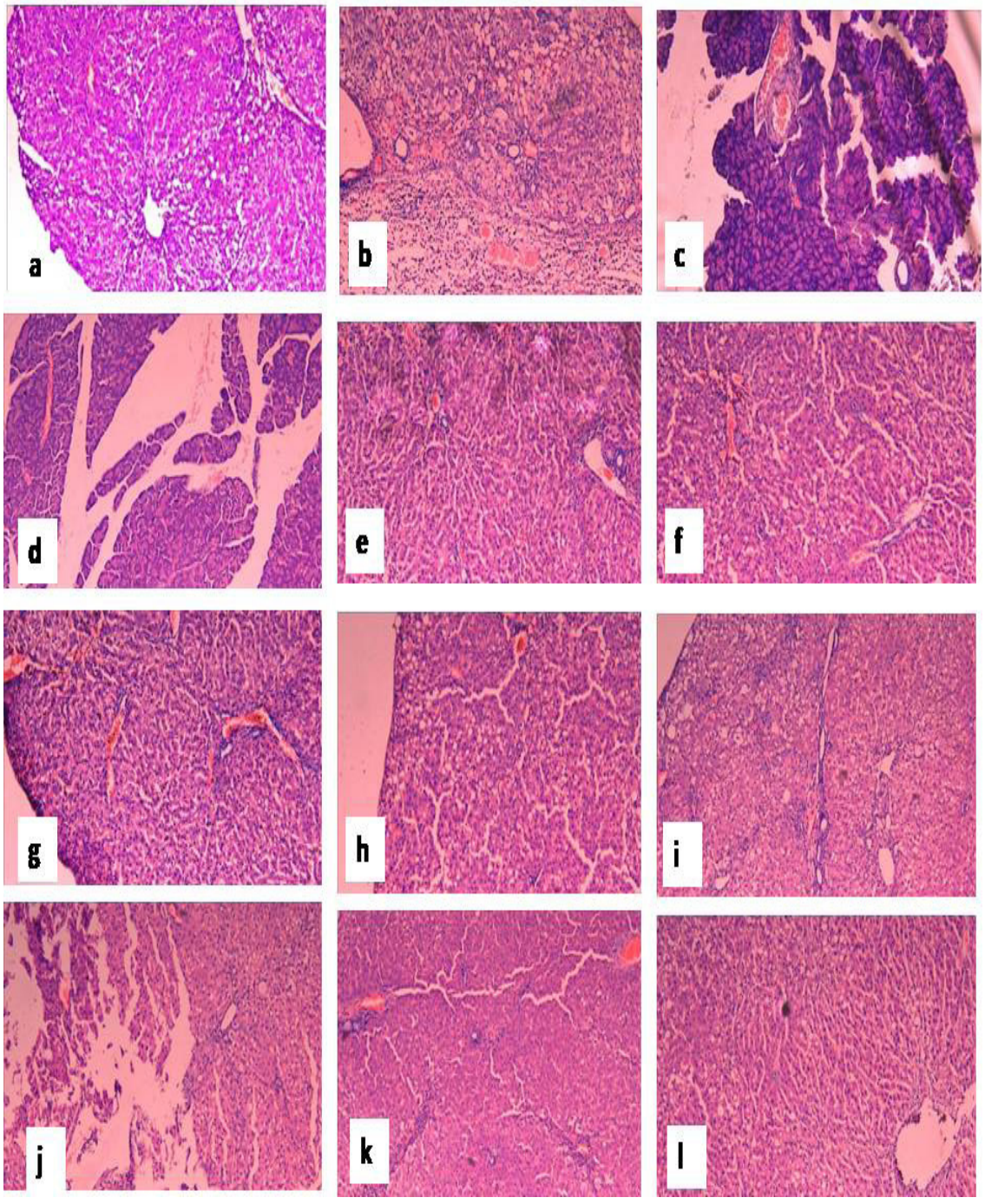

Figure 1. Photomicrograph (10X) of liver section treated with different test dose, standard drug, positive control and negative control groups.
a: Positive control group, administered with vehicle, **b**: Group treated with standard drug, **c**: Negative control group, Induced with Streptozotocin, **d**: Group treated at dose of 10 mg/kg body weight with F12, **e**: Group treated at dose of 20 mg/kg body weight with F12, **f**: Group treated at dose of 50 mg/kg body weight with F12, **g**: Group treated at dose of 10 mg/kg body weight with F16, **h**: Group treated at dose of 20 mg/kg body weight with F16, **i**: Group treated at dose of 50 mg/kg body weight with F16, **j**: Group treated at dose of 10 mg/kg body weight with F48, **k**: Group treated at dose of 20 mg/kg body weight with F48, **l**: Group treated at dose of 50 mg/kg body weight with F48.

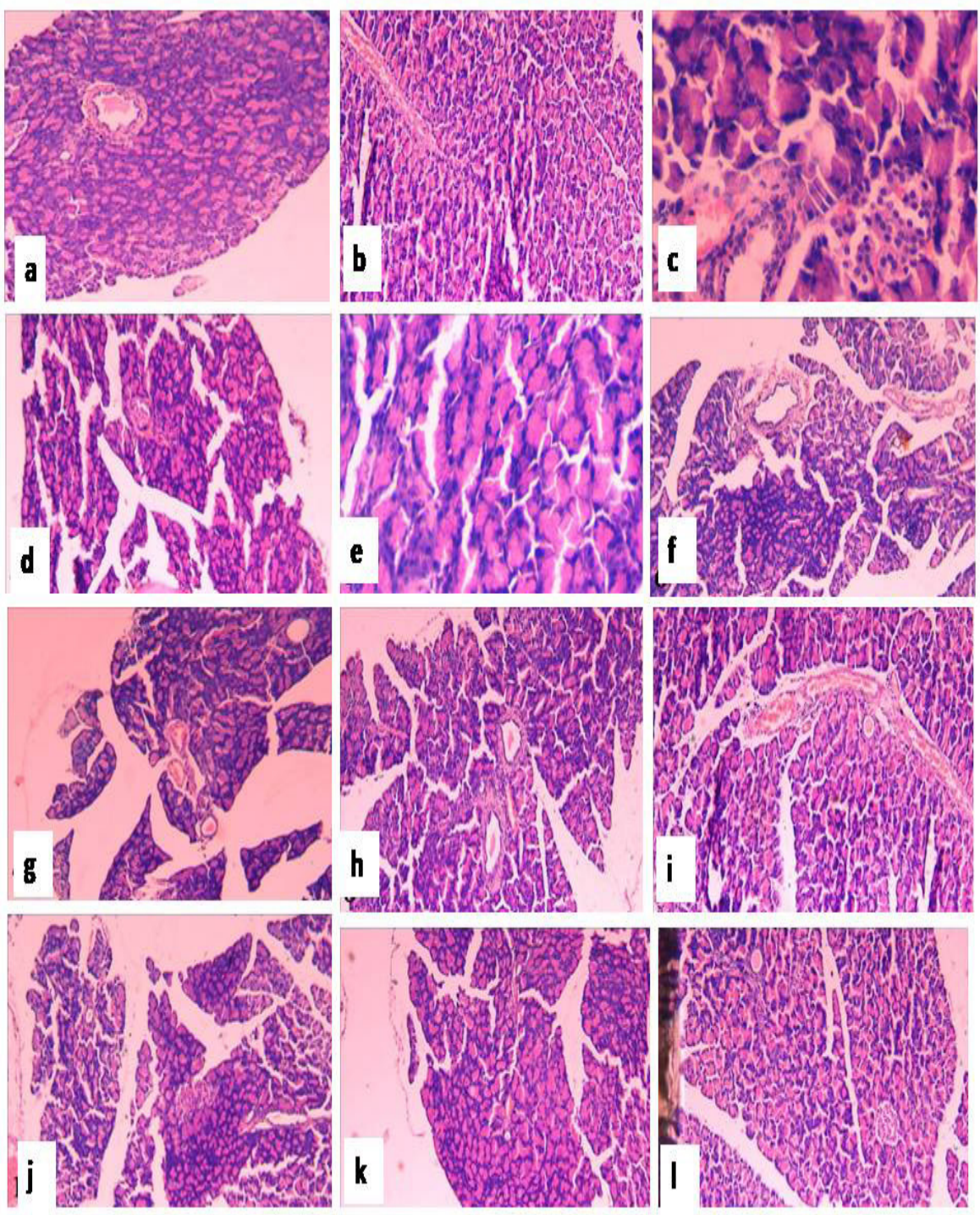

Figure 2. Photomicrograph (10X) of pancreas section treated with different test dose, standard drug, positive control and negative control groups.
a: Positive control group, administered with vehicle, **b**: Group treated with standard drug, **c**: Negative control group, Induced with Streptozotocin, **d**: Group treated at dose of 10 mg/kg body weight with F12, **e**: Group treated at dose of 20 mg/kg body weight with F12, **f**: Group treated at dose of 50 mg/kg body weight with F12, **g**: Group treated at dose of 10 mg/kg body weight with F16, **h**: Group treated at dose of 20 mg/kg body weight with F16, **i**: Group treated at dose of 50 mg/kg body weight with F16, **j**: Group treated at dose of 10 mg/kg body weight with F48, **k**: Group treated at dose of 20 mg/kg body weight with F48, **l**: Group treated at dose of 50 mg/kg body weight with F48.

Instrumental Characterization of Selected Potent Compounds

Based on the data generated from the *in vivo* studies conducted on the compounds isolated from MECP, the compounds F48 and F16 were found to be most potent, which can be comparable with that of the standard drug.

Spectral data obtained for compound F48 and F16 from different instrumental techniques are represented in Figure 3.

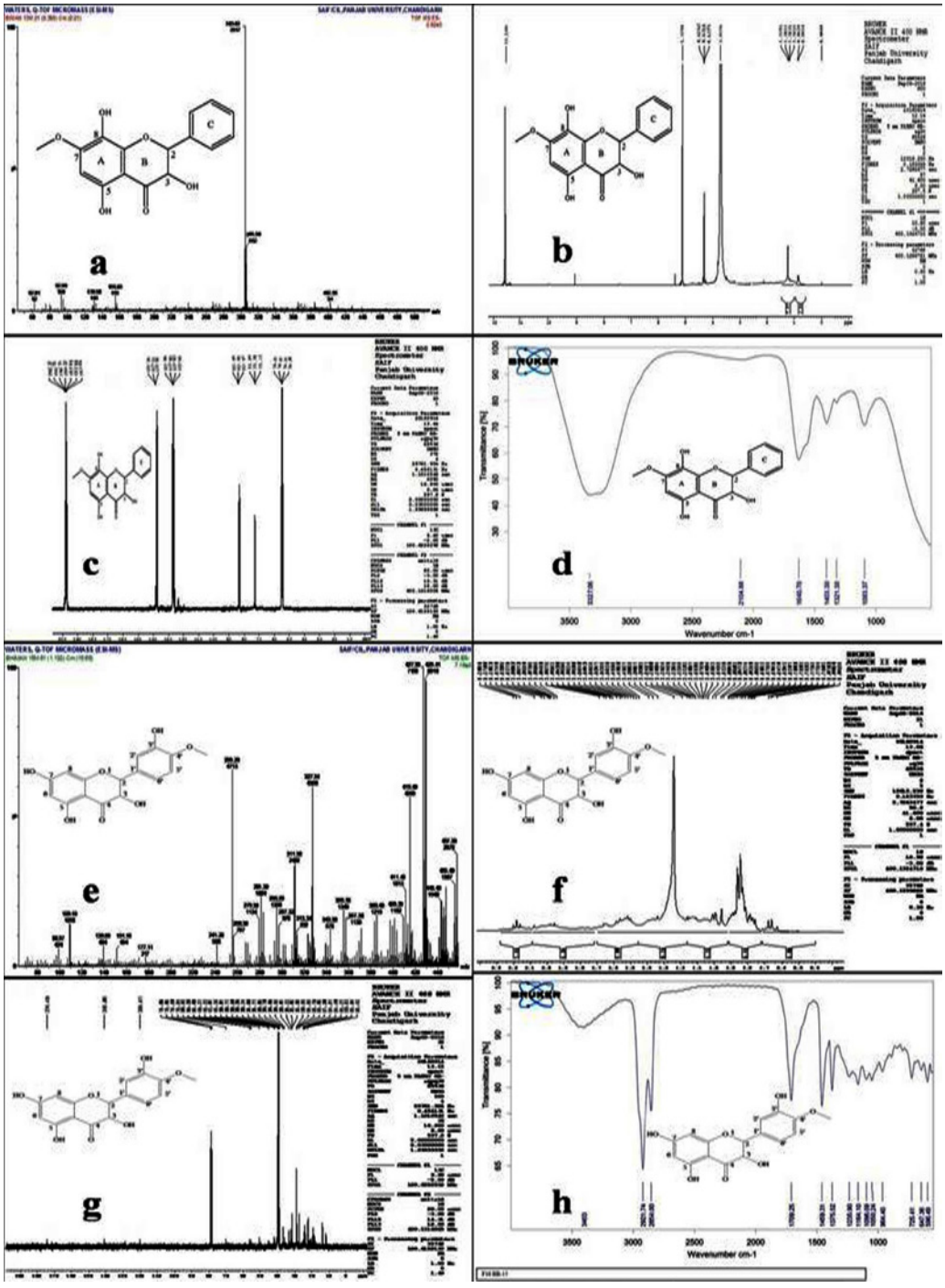

Figure 3. Instrumental characterization of compound F48 and F16.
a: Mass spectra of compound F48; **b**: Proton NMR spectra of compound F48; **c**: Carbon NMR spectra of compound F48; **d**: FTIR spectra of compound F48; **e**: Mass spectra of compound F16; **f**: Proton NMR spectra of compound F16; **g**: Carbon NMR spectra of compound F16; **h**: FTIR spectra of compound F16

Structure Interpretation of Isolated Compounds

Compound F48

Compound F48 is pale yellow colored powder with a melting point range is 226-228 °C. Molecular formula of the compound F48 is deduced as $C_{16}H_{14}O_6$ [(M+H+) peak at m/z 303.08]. The 1H NMR spectrum of displayed signals of two methine doublets at 5.17 (C-2) and 4.62 (C-3), a methoxy group at 3.82 and a hydroxyl group at 11.59(due to strong H-bonding). The 13CNMR spectrum of compound also displayed two methines at 82.9 (C-2) and 71.8(C-3), a methoxy group at 56.1 (OMe), a conjugated ketone at 198.3 and the signals of two aromatic rings at137.3 (ring A) and 127.0 (ring C) respectively. IR spectrum displayed absorption due to a chelated carbonyl which appeared at 1640 cm^{-1} and a hydroxyl appeared at 3327 cm^{-1}. This suggested that compound is a flavanone with an unsubstitued B ring with IUPAC name 3, 5, 9-trihydroxy-7-methoxy-2-phenyl-2,3-dihydrochromen-4-one or 3,5,8-trihydroxy-7-methoxy flavanone. The structure is presented in Figure 3.

Compound F16

Compound F16 is light yellow colored powder with a melting point range is 225-226 °C. Molecular formula of the compound F16 is deduced as $C_{16}H_{14}O_7$ [(M+H+) peak at m/z 318.07]. The 1H NMR spectrum of displayed signals of two methine doublets at 5.30 (s, 1H, aryl OH), 5.24 (d, 1H, J=4 Hz, >CH-, C-2) and 5.10 (d, 1H, J=4 Hz, >CH-, C-3) and a methoxy group at 2.19 (s, 3H, OCH_3; due to strong H-bonding). The 13CNMR spectrum of compound also displayed one methine at 70.04 (C-3, >CH-), a methoxy group at 56.20 (OMe), a conjugated ketone (>C=O, conj.) at 174.45 and the signals of two aromatic rings at140.98 (aryl-C, ring A) and 120.27 (aryl-C, ring C) respectively. IR spectrum displayed absorption due to a chelated carbonyl (>C=O, conj.) which appeared at 1709.25 cm^{-1} and a hydroxyl group at 3403.05 cm^{-1} (O-H, bonded), absorption due to methoxy appeared at 2921.74 cm^{-1}, 2854.00 cm^{-1} (C-H, OCH_3). This suggested that compound is a flavanone with an unsubstitued B ring with IUPAC name 3,5,7-Trihydroxy-2-(3′-hydroxy-4′-methoxy phenyl)-2,3-dihydrochromen-4-one or [(3,5,7-Trihydroxy-3′-hydroxy-4′-methoxy) flavanone]. The structure is presented in Figure 3.

CONCLUSION

Isolation of phytoconstituents from MECP was initiated using exhaustive column chromatography. Six compounds were successfully sequestered with the help of recolumn, purification and TLC characterization. Three of the six compounds *viz*. F12, F16 and F48 showed promising *in vitro* results. IC_{50} values of compounds were taken into consideration for selecting the potent compounds and two *in vitro* methods were performed i.e. DNSA inhibition assay and Iodine-Starch method. IC_{50} values of F12, F16 and F48 were found to be 10.16, 4.30 and 3.25 µg/ml respectively as per DNSA inhibition assay and that by Iodine-Starch method were found to be 11.69, 8.9 and 5.57 µg/ml respectively. Compound F48 exhibited maximum activity followed by compound F16 and compound F12. Compounds F48, F16 and F12 were therefore considered for *in vivo* anti diabetic activity using three dose regimens *viz*. 10, 20 and 50 mg/kg body weight respectively. Compounds isolated from MECP were found to alter the biomarker levels and the 50 mg/kg/ body weight dose closely resembles to that of the standard group in successfully controlling the elevated cholesterol and triglyceride levels. The groups F48.3 and F16.3, treated with 50mg/kg dose significantly reversed the STZ induced diabetic parameters (increased blood glucose level, altered plasma profile and histoarchitecture of the pancreatic and hepatic cells), which is also evident from the histopathology data that is comparable with the standard drug. The compoundsF48 and F16 isolated from MECP were hence considered for further instrumental characterization for identification and validation of components responsible for the said biological activity. IUPAC name of the compounds were deduced based on different instrumental techniques and F48 was deduced as 3,5,9-trihydroxy-7-methoxy-2-phenyl-2,3-dihydrochromen-4-one or 3,5,8-trihydroxy-7-methoxy flavanone and compound F16 the IUPAC name was deduced as 3,5,7-Trihydroxy-2-(3′-hydroxy-4′-methoxy phenyl)-2,3-dihydrochromen-4-one or [(3,5,7-Trihydroxy-3′-hydroxy-4′-methoxy) flavanone].

The study findings were thus successful in identifying two flavanones as most potent compounds in exhibiting antidiabetic activities thereby validating the traditional belief associated with the use of *Costus pictus* D. Don.

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